British Heart Journal, 1974, **36**, 167–170.

Fluorescent anti-heart IgM and raised levels of serum IgM in newborns with congenital heart diseases[1]

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Thirty-five per cent (13 of 37) of newborns, studied at less than 2 weeks of age, with congenital heart lesions, had abnormally high serum levels of both IgM (> 20 mg/100 ml) and anti-heart IgM demonstrable by a fluorescent technique. An association between the raised serum IgM, reflecting in utero *infection, and the congenital heart defects may be deduced. Possible explanations of the presence of anti-heart IgM in the sera of these newborns include modification of the antigenicity of the developing heart with teratogenic effect produced by the invading organism, but not the anti-heart IgM; and direct teratogenic influence of the anti-heart antibody of fetal origin raised against both the invading organism and shared (or modified) antigens in the host.*

Of the dozen viruses which can reach the fetus, 3 or possibly 4 have been associated with congenital malformations to the extent that they may be considered to be teratogenic (rubella virus, cytomegalovirus, *Herpes virus hominis*, and possibly Coxsackie B viruses). Raised levels of IgM have been found in the cord blood of newborns with congenital rubella and cytomegalic inclusion disease and have been taken as evidence of *in utero* infection with fetal synthesis of antibody (Sever, 1969), because the size of the IgM macroglobulin does not permit placental transfer from the mother to the fetus under normal conditions.

The mechanism by which viruses may cause maldevelopment are not completely understood. Rubella virus in the human is probably teratogenic through a direct virus-host cell interaction. For a virus to cause maldevelopment the infection must occur at a vulnerable period of embryogenesis, usually in the first trimester. And for a virus to cause fetal synthesis of IgM, immunologically responsive tissue must be present. The development of thymic and lymphoid tissues occurs at about the same time as development of the heart.

Antibodies against kidney (Brent, 1964) have been documented to have a generalized teratogenic effect producing multiple anomalies in experimental animals. Anti-heart antibody appears to have both a generalized and organ-specific effect (Nora, 1971) in that there is a significantly higher frequency of cardiovascular anomalies produced by anti-heart antibody than by anti-kidney antibody. Anti-heart antibody is detectable by immunofluorescent staining techniques, which have also shown cross-reaction between antigens of heart of several mammalian species, human skeletal muscle, and the phylogenetically distant Group A streptococci (Kaplan, Meyeserian, and Kushner, 1961; Kaplan and Meyeserian, 1962).

Received 30 July 1973.

[1] Supported by a grant from the American Heart Association and a grant from the National Heart and Lung Institute.

Subjects and methods

Blood was obtained from newborns, 2 weeks of age or less, with congenital heart lesions, who have been studied at cardiac catheterization, to look for increases in IgM and to determine the presence in their serum of anti-heart IgM by immunofluorescent staining. A control group of 30 newborns without heart defects was obtained from the same medical centre population and matched for race, age, and socioeconomic level.

Quantitative IgM levels were obtained by a modification of the single radial diffusion technique of Mancini, Carbonara, and Heremans (1965) using a 1:30 final dilution of antiserum in 2 per cent agar, incubating in 1·5 mm wells a 1:6 dilution of patient's serum in phosphate buffered saline for 18 hours at 24°C, then dipping in 2 per cent acetic acid for 15 seconds. Multiple dilutions of duplicate controls of standardized and stabilized human serum Behringwerke were read by a millimetre eyepiece against duplicate serum samples of the newborns.

The fluorescein-conjugated anti-IgM was supplied by Rossen and prepared by the bentonite immunoadsorbent technique of Reisberg, Rossen, and Butler

(1970). Appropriate blocking and absorption experiments were performed on the fluoresceinated reagents to insure their specificity. Methods of preparation of these reagents and controls which have been previously presented (Reisberg *et al.*, 1970; Rossen *et al.*, 1971) will not be repeated in detail.

Sheep antisera to human IgM was prepared by immunization with the serum from a patient with macroglobulinaemia. The human serum was purified by a combination of sodium sulphate precipitation, gel filtration through Sephadex G-200, and starch block zone electrophoresis. It was shown to be free of contaminating serum proteins by immunoelectrophoresis at a concentration of 3 mg/ml. The hyperimmunization of the sheep with this human IgM was achieved using Freund's complete adjuvant. The antisera to human IgM was shown to be specific for the immunizing antigen by immunoelectrophoresis. Specific bentonite immunoadsorbents were prepared and fluorescein conjugation achieved by the method of Reisberg *et al.* (1970). Antibody specific for μ-chain was isolated with IgM-bentonite adsorbent and sequentially adsorbed with excess IgG or Cohn Fr. II, IgA, lambda, and kappa adsorbents.

Frozen tissue sections of normal human heart shown to be free of globulin deposition were cut to 4 to 6 μm, fixed in 95 per cent ethanol, rehydrated with saline buffered at *p*H 7·4, placed on slides with full-strength patient's serum sufficient to cover the tissue, incubated for 30 minutes at room temperature in a moist chamber, washed with buffered saline, and dried. Fluorescein isothiocyanate labelled anti-IgM (prepared as above) was then applied to the tissue, followed by incubation at room temperature for 30 minutes. The slides were again washed with phosphate buffered saline. Negative controls of normal heart muscle which did not react to fluoresceinated antibody and positive controls of fluorescein stained rejected human cardiac allograft (previously demonstrated to have globulin deposition) were interspersed randomly with the slides of interest. The coded slides, in duplicate from each patient, and the positive and negative controls were examined each day by the same observer for the presence or absence of fluorescence in sarcolemma, muscle fibres, interstitial spaces, connective tissue, and blood vessels. The intensity of fluorescent staining was graded 1+ to 4+ and the observer had no knowledge at the time of reading of the origin of the tissue sections. The slides were studied with a Leitz Ortholux microscope fitted with Corning No. 7–60 and No. 7–57 excitation filters and Kodak K430 and K150 barrier filters. Tissues were examined on two separate occasions before the results of the study were decoded.

Results

As recorded in Table 1, 16 of 38 newborns (41%) aged 1 to 12 days who had congenital heart defects had increases of IgM greater than 20 mg/100 ml. Of the 30 newborns (aged 1 to 12 days) in the control group, only 2 had IgM levels greater than 20 mg/100 ml. Only increases which persisted in samples taken one week after the original samples were accepted as valid. Increases of IgM in these newborns may be assumed to reflect fetal synthesis of antibody in response to *in utero* infection. Comparing the congenital heart group with normal newborns by χ^2 with respect to raised IgM yields a probability of $<0{\cdot}01$.

TABLE 1 *Quantitative serum levels of IgM in newborns with congenital heart lesions compared with newborns from the same medical centre population who were demonstrably free of cardiovascular or other anomalies*

	IgM > 20 mg/100 ml	*IgM < 20 mg/100 ml*	*Totals*
Congenital heart	16	22	38
Control	2	28	30
Totals	18	50	68

In Table 2, one patient has been eliminated who had a quantitative IgM determination (>20 mg/100 ml), but on whom no fluorescent anti-heart IgM study was obtained. This Table reveals that 13 newborns (of the 15 congenital heart patients who had raised levels of IgM and a fluorescent anti-heart antibody study) also had fluorescent anti-heart IgM in their sera. None of the 30 control patients had anti-heart IgM, though 2 control patients had raised serum levels of IgM. This difference is significant at the 0·001 level. Two newborns who had congenital heart lesions and increases of IgM did not have evidence of anti-heart IgM by fluorescent studies.

Table 3 records the heart lesions and the ages of the patients at the time of the study. There is nothing unusual about the types of lesions encountered. These are typical of the defects requiring cardiac catheterization before 2 weeks of age. What is noteworthy is that transposition of the great vessels appears 4 times and truncus arteriosus once, indicating that the teratogenic insult

TABLE 2 *Fluorescent anti-heart IgM in sera of 37 newborns with congenital heart defects who had simultaneous quantitative serum IgM determinations compared with same control group in Table 1*

	Positive	*Negative*	*Totals*
Congenital heart	13	24	37
Control	0	30	30
Totals	13	54	67

TABLE 3 *Cardiovascular anomalies and ages of 13 newborns who had both raised serum IgM and fluorescent anti-heart IgM*

Anomaly	*Age (dy)*	*IgM (mg/100 ml)*	*Fluor. IgM*
Transposition great arteries	1	58	4+
Transposition great arteries	2	55	2+
Transposition great arteries	2	23	3+
Transposition great arteries	3	60	3+
Pseudotruncus	1	47	2+
Pseudotruncus	1	26	3+
Pseudotruncus	3	42	2+
Pulmonary atresia (type I)	3	24	3+
Tricuspid atresia	2	28	2+
Coarctation of aorta (VSD and PDA)	9	100	4+
VSD and PDA	11	55	4+
Pulmonary valve stenosis	12	46	3+
Truncus arteriosus	6	28	3+

VSD = ventricular septal defect; PDA = persistent ductus arteriosus.

could not have occurred later than the fifth week of gestation in these particular newborns. The serum samples of all but three of the patients were obtained during the first week of life and no patient in either the experimental or control groups was older than 12 days. None of the specimens of patient in this study who had fluorescent anti-heart IgM was interpreted as having an intensity less than 2+. Significant sarcolemmal binding was a criterion of a 4+ reaction. Though the highest serum level of IgM was associated with a 4+ fluorescent reaction, there was no clear correlation between serum IgM levels and the intensity of fluorescence.

Discussion

There are various possible interpretations of these findings. To begin with there is a highly significant association between evidence for *in utero* infection and the cardiovascular malformations found in the newborns, though there is no demonstrable causal relation. In the case of rubella, the association between viral infection and congenital heart disease is well established. The high frequency with which high levels of IgM were found in these newborns is provocative and justifies further investigation of congenital heart patients in a search for specific viral agents.

The demonstration of fluorescent anti-heart IgM in the sera of 13 newborns with congenital heart defects who had raised serum levels of IgM may mean that a presumed *in utero* infection continued through the periods of development of the heart and thymic-lymphoid system and eventually called forth an antibody response. This was detectable as anti-heart IgM, though neither the anti-heart antibody nor the *in utero* infections played teratogenic roles in the maldevelopment of the cardiovascular systems of these newborns.

A more positive and equally plausible explanation is that the infection played a teratogenic role through a mechanism such as direct virus-host cell interaction, but that the anti-heart IgM itself had no teratogenic influence. A corollary of this could be that the infecting organism modified the antigenicity of the developing heart so that when the fetus became immune-competent, it recognized the heart as 'non-self' and raised antibody against it (anti-heart IgM). This explanation would be most consistent with available evidence, and the presence of anti-heart IgM would add further aetiological significance to the high IgM levels.

Yet another explanation is that the anti-heart antibody, raised against the invading organism and shared, or modified, antigens in the host, was teratogenic to the developing heart. Present limitations in our knowledge of immunological development in the human permit us considerable latitude for speculation. The specificity of anti-heart antibody for heart alone has not been demonstrated by us or other investigators. Cross-reaction with skeletal muscle has been shown by Kaplan and Meyeserian (1962) and was found by us in a study of cardiac transplant patients using the same conjugates and methods employed in this investigation (Rossen *et al.*, 1971). There is experimental evidence for generalized as well as organ-specific teratogenicity of antibody raised against kidney, heart, and more discrete protein components of tissues (Langman, 1964). Our experiments with anti-heart antibody in the mouse show it to be one of the most potent teratogens we have used to produce cardiovascular maldevelopment and suggest that anti-heart anti-

body has greater specificity for the heart than anti-kidney antibody (Nora, 1971).

Small lymphocytes have been seen to appear in the peripheral blood of the human fetus as early as 7 weeks' gestation (Playfair, Wolfendale, and Kay, 1963). During the 7th week of gestation lymphocytes also migrate to the thymic primordia (Valdes-Dapena, 1957). Immune competence of these cells as early as this in the human has not been demonstrated, but immune responsiveness in the sheep has been documented even before the appearance of thymic and lymphoid tissue (Silverstein, 1969). Given the demonstrable teratogenicity of anti-heart antibody to the developing mammalian heart, the presence of potentially immune competent tissue in the human fetus at a stage of organogenesis when anti-heart antibody could exercise teratogenic influence, and the detection of both raised serum levels of IgM and fluorescent anti-heart IgM in newborns with congenital heart lesions, the proposal that anti-heart antibody of fetal origin may be teratogenic to the developing human heart is a possibility which should be investigated further.

Certainly, virus-host cell interaction is most likely to be the teratogenic mechanism of cardiovascular maldevelopment in our patients. However, autoimmune host reponse to an infecting organism remains a possible alternative in some patients.

We wish to thank Dr. Roger Rossen, not only for supplying the fluorescein conjugate, but for his advice and assistance throughout the study.

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